RESEARCH COMMUNICATION

The bioactive phospholipid lysophosphatidic acid is released from activated platelets

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Lysophosphatidic acid (LPA) is a water-soluble phospholipid with hormone-like and growth-factor-like activities. LPA activates a putative G-protein-coupled receptor in responsive cells, but the natural source of exogenous LPA is unknown. Here we show that LPA is present in mammalian serum in an active form (bound to albumin) at concentrations of 1–5 μM, but is not detectable in platelet-poor plasma, suggesting that LPA is produced during blood clotting. We find that thrombin activation of platelets prelabelled with [^{32}P]P_i results in the rapid release of newly formed [^{32}P]LPA into the extracellular environment. We conclude that LPA is a novel platelet-derived lipid mediator that may play a role in inflammatory and proliferative responses to injury.

INTRODUCTION

Apart from playing a key role in the early steps of glycerolipid biosynthesis [1], lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol 3-phosphate) evokes a great variety of biological effects when added to responsive cells [2]. For example, exogenous LPA causes platelet aggregation [3,4], smooth-muscle contraction [5,6], neuronal cell rounding and neurite retraction [7] and, in fibroblasts, cell proliferation [8,9]. Current evidence strongly suggests that LPA evokes its multiple effects through activation of a specific G-protein-coupled receptor, with consequent stimulation of phospholipases C and D and inhibition of adenylate cyclase [8,10–12]. A candidate LPA receptor was recently identified by photoaffinity labelling in various LPA-responsive cell types [13]. However, the natural source of LPA remains unknown to date.

LPA is rapidly produced in thrombin-activated platelets [14–17], apparently in large part through deacylation of newly formed PA [17,18]. A PA-specific phospholipase A_2 has been detected in platelets [18], but its function has been regarded primarily as a mechanism for the production of arachidonate and its bioactive metabolites. Since LPA is fairly water-soluble (critical micelle concentrations 70 μM–1 mM [10,19]), it is possible that endogenously produced LPA, in common with arachidonate metabolites, is released from platelets into the extracellular space to act as a local mediator. The present study was undertaken to examine this possibility. We report that LPA is rapidly released from human platelets upon thrombin stimulation and is present in serum (bound to albumin) at physiologically active concentrations, but not in platelet-poor plasma. These results assign a physiological role to LPA as a platelet-derived lipid mediator that may participate in natural wound-healing processes.

MATERIALS AND METHODS

Materials

Lipid standards were obtained from Serdary Research Laboratories (London, Ont., Canada). Human α-thrombin, bradykinin, BSA (Fraction V, fatty-acid-free) and prostaglandin E_1 were from Sigma; silica gel 60 plates were from Merck; Indo-1 acetoxymethyl ester was from Molecular Probes (Eugene, OR, U.S.A.). Fetal-calf serum (FCS; various batches) was from GIBCO. Carrier-free [^{32}P]P_i was obtained from Amersham. 1-Oleoyl-[^{32}P]LPA was synthesized as described [13].

Platelet preparation

Human blood from healthy volunteers was anticoagulated with acid/citrate/dextrose as described [20,21]. Platelet-rich plasma was obtained by centrifugation (120 *g* for 15 min) and immediately mixed with prostaglandin E_1 (100 nM) to suppress platelet activation. Platelets were collected by centrifugation (1200 *g* for 10 min); after two additional centrifugation steps (8000 *g* for 5 min) the supernatant was considered 'platelet-poor' plasma. The platelet pellet was washed three times in a buffer containing 36 mM citric acid, 103 mM NaCl, 4 mM KCl, 5 mM EDTA, 5.6 mM glucose, 0.35% (w/v) BSA and 100 nM prostaglandin E_1 (pH 6.5). Platelet counts were determined in a Coulter counter.

Platelet activation and lipid extraction

Before activation, platelets (2×10^9 cells/ml) were labelled for 4 h in phosphate-free Eagle's Minimum Essential Medium (Flow Laboratories) containing 1 mCi/ml [^{32}P]P_i, in the absence or the presence of 10 mM EDTA. Platelets were activated by stirring with 1 unit/ml human α-thrombin in either the absence or the presence of 10 mM EDTA. After stimulation, cells were pelleted by centrifugation for 2 min at 3000 *g* and resuspended in water. Supernatants were further cleared from cell debris by centrifugation at 8000 *g* for 5 min. Lipids from cells and supernatants were extracted separately with butan-1-ol (see below). For serum lipid extraction, FCS (four different batches; freshly thawed) and human serum (from two volunteers) was diluted 10-fold in water. All solutions were made 0.02 M in acetic acid and extracted with 0.5 vol. of butan-1-ol as described [22,23]. In the case of serum,

Abbreviations used: LPA, lysophosphatidic acid; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; FCS, fetal-calf serum.

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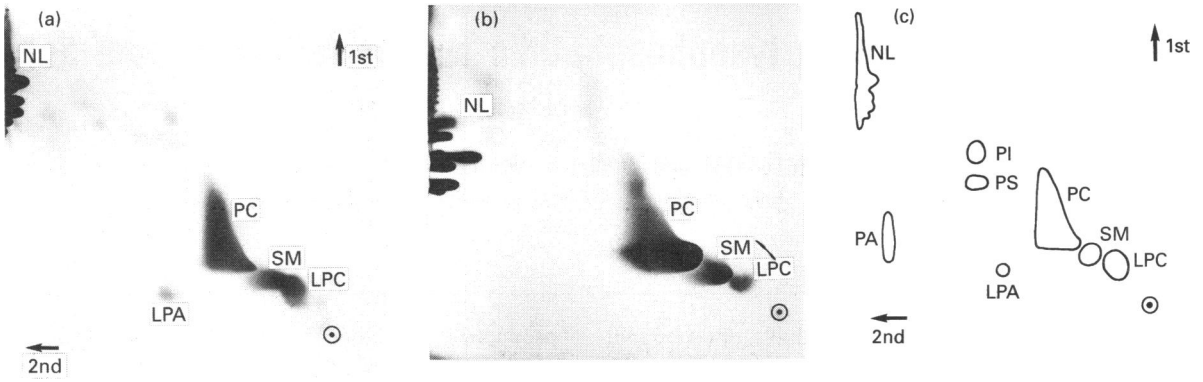

Figure 1 Separation of serum lipids by two-dimensional t.l.c.

Lipids were extracted from FCS (**a**) or from human platelet-poor plasma (**b**), separated by two-dimensional t.l.c. and revealed by iodine staining as described in the Materials and methods section. Lipids were identified by co-migration with authentic standards as indicated in (**c**). Abbreviations: NL, neutral lipids; LPC, lyso-PC; ⊙, origin. When extracted from the t.l.c. plate, only the spot co-migrating with authentic LPA showed biological activity (see the text).

the two-phase system was frozen after thorough mixing and thawed again to obtain better separation of the two liquid phases and the protein interphase. After centrifugation, the butan-1-ol phase was removed and the water phase was extracted once again. Butanol fractions were collected and washed twice with 1 vol. of butan-1-ol-saturated water. The butan-1-ol phase was dried under nitrogen and analysed on a two-dimensional t.l.c. system as described below. Recovery of added tracer [^{32}P]LPA was 98%.

Phospholipid analysis

Phospholipids were separated by two-dimensional t.l.c. [24] on silica gel 60 plates and the solvent systems chloroform/methanol/7 M ammonia (12:12:1, by vol.; two successive runs) and subsequently chloroform/methanol/acetic acid/water (25:15:4:2, by vol.). Serum- and platelet-derived lipids were detected by iodine staining and autoradiography, respectively, and identified by co-migration with non-radioactive marker lipids. Radioactive phospholipids were scraped off the plate and prepared for liquid-scintillation counting. The counts were normalized with respect to the total amount of ^{32}P-labelled phospholipids.

We note that, under the labelling conditions used, the total radioactivity of [^{32}P]phosphatidic acid ([^{32}P]PA) represents an adequate direct measure for its mass (e.g. [25]). It seems plausible to assume that the same holds for [^{32}P]LPA, although direct proof for that notion awaits further study. In platelets, not all phospholipids are labelled equally well with ^{32}P. Whereas (L)PA and phosphatidylinositol (PI) are rapidly and efficiently labelled to isotopic equilibrium [25] and thus are readily detectable, the major phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) are poorly labelled, if at all. Poor ^{32}P-labelling of PC, PE and SM in platelets is probably due to the relatively low rates of synthesis *de novo* of their precursors [26,27].

Serum phospholipids were scraped into vials and quantified by the phosphorus-content assay as described [28,29]. For acyl-chain analysis of serum phospholipids, fatty acid methyl esters were generated by heating the silica gels with 4% HCl in methanol at 80 °C for 4 h. The methyl esters were analysed in a gas chromatograph (Carlo Erba Strumentazione, model HRGC 5300) equipped with a flame ionization detector. The stationary phase was 10% CP-Sil 58 in a glass column (2500 cm × 0.32 cm).

Measurement of cytoplasmic free Ca^{2+}

Confluent human A431 cells, grown on glass coverslips, were loaded with the fluorescent Ca^{2+} indicator Indo-1 and Ca^{2+}-dependent fluorescence was monitored as described [10]. A431 cells were used because they are highly responsive to exogenous LPA with regard to Ca^{2+} mobilization ([10], and results not shown).

Gel-filtration chromatography

BSA/[^{32}P]LPA mixtures were fractionated on a Sephadex G-50 gel-filtration column as explained in the Results and discussion section. The column was equilibrated and run in phosphate-buffered saline (150 mM NaCl, 3.3 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4; pH 7.2). Fractions (0.5 ml) were collected and processed for protein determinations and/or liquid-scintillation counting. Relative protein concentration was measured by u.v. absorbance at 280 nm.

RESULTS AND DISCUSSION

LPA as a bioactive serum constituent

If LPA is released from activated platelets, it may be detectable in whole serum. We extracted FCS with butanol and analysed the lipid fraction by a two-dimensional t.l.c. system [24] that allows LPA to be separated from other polar lipids with a high degree of resolution. Figure 1(a) shows the result of a typical experiment in which LPA is clearly detected and identified as a normal serum constituent, based on its co-migration with authentic standards (Figure 1c). Four other experiments, using different batches of FCS, gave similar results. Using the same extraction protocol, we also detected LPA in freshly isolated human serum (results not shown), but not in human platelet-poor plasma (Figure 1b). Quantitative phosphorus analysis of the scraped LPA spots yielded an average serum LPA concentration of approx. 2 μM (range 1–5 μM; four determinations yielding values of 1.1, 1.2, 2.0 and 4.8 μM). Qualitative analysis by g.l.c. revealed that the major species of LPA in FCS

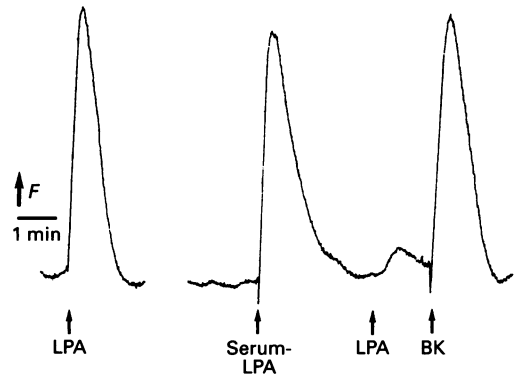

Figure 2 Effect of serum-derived LPA on Ca^{2+} mobilization in A431 cells

Typical time courses relative changes in cytoplasmic free Ca^{2+} in human A431 cells stimulated with synthetic 1-oleoyl-LPA (500 nM; left trace) and, in a separate experiment, with serum-derived LPA (Serum-LPA; approx. 500 nM), synthetic 1-oleoyl-LPA (LPA: 500 nM) and bradykinin (BK; 1 μM), from left to right, as indicated. Note desensitization of the response to synthetic LPA, but not bradykinin, by prior addition of serum-derived LPA. *F* denotes Ca^{2+}-dependent indo-1 fluorescence.

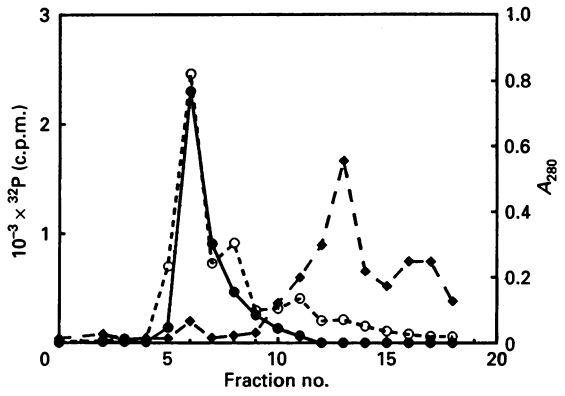

Figure 3 Co-fractionation of [^{32}P]LPA with serum albumin

BSA (●), 1-oleoyl-[^{32}P]LPA (◆), or BSA preincubated for 20 min with equimolar amounts of 1-oleoyl-LPA and trace amounts of [^{32}P]LPA (○) was applied to a Sephadex G-50 column and eluted with phosphate-buffered saline (see the Materials and methods section). Fractions (0.5 ml) were collected and assayed for [^{32}P]LPA content (c.p.m.) and/or BSA concentration (u.v. absorbance at 280 nm).

are palmitoyl-, stearoyl-, oleoyl- and arachidonoyl-LPA (T. Hengeveld and K. Jalink, unpublished work); these fatty acid species are very similar to those produced in activated platelets [17].

Figure 2 shows that when serum LPA is extracted from the t.l.c. plate and applied to Indo-1-loaded A431 cells, an immediate Ca^{2+} transient ensues which is indistinguishable from that evoked by synthetic long-chain LPA [10] or whole serum [30]. Prior addition of serum-derived LPA completely desensitizes the response to subsequent addition of synthetic 1-oleoyl-LPA, but leaves the response to the peptide bradykinin unaltered (Figure 2). It thus appears that serum-borne LPA, like synthetic LPAs, can serve as a potent Ca^{2+}-mobilizing agonist.

van der Bend et al. [13] recently showed that photoreactive LPA can bind to three distinct serum proteins, namely BSA (68 kDa) and, to a lesser extent, 28 kDa and 15 kDa proteins. We applied a mixture of BSA and 1-oleoyl-LPA containing tracer amounts of [^{32}P]LPA to a gel-filtration column and found that LPA co-elutes with BSA (Figure 3). Furthermore, when 1-oleoyl-LPA was mixed with BSA (molar ratio 2:1), its biological activity (as determined by Ca^{2+} mobilization and neurite retraction) was not detectably reduced; extensive dialysis (30 kDa cut-off) of the LPA/BSA mixture against water did not result in loss of activity, whereas dialysis of a BSA-free LPA solution completely removed activity (results not shown). Taken together, these results indicate that LPA binds tightly to albumin and that albumin-bound LPA is biologically active. Support for this notion comes from a recent study by Ridley and Hall [31], who inferred that the stress-fibre-inducing activity of serum, as observed in fibroblasts, is likely to be due to albumin-bound LPA.

Release of LPA from activated platelets

We next examined whether LPA is released from platelets when they are activated under physiological conditions. Freshly isolated human platelets were prelabelled with [^{32}P]P_i and then stimulated with human thrombin for 10 min. followed by lipid extraction of cells and medium. As demonstrated in Figure 4 and

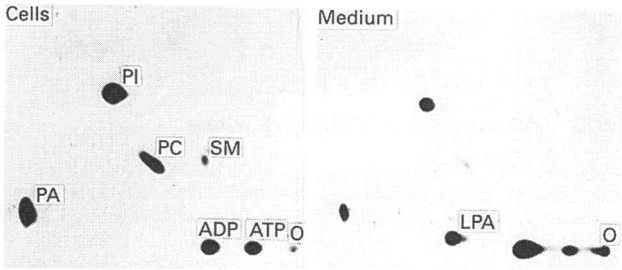

Figure 4 LPA release by thrombin-stimulated platelets

Cells were labelled with [^{32}P]P_i, stimulated for 10 min with thrombin and separated from the medium by centrifugation, as described in the Materials and methods section. Lipids were extracted from both the cell pellet and medium, and analysed by two-dimensional t.l.c. Labelled spots were identified by co-migration with unlabelled markers. The autoradiogram shows phospholipids present in the cells and medium, as indicated. Abbreviation: O, origin. The appearance of labelled PA and PI in the medium is due to contaminating membrane fragments (see also Table 1). Note the appearance of radiolabelled ADP and ATP in the medium (these spots may also contain GDP and GTP, respectively).

Table 1, thrombin-activated platelets rapidly release a large part of their newly formed LPA into the extracellular environment, whereas newly generated PA (like PC and PI) remains associated mainly with the cells. In agreement with previous reports [16,17], we detected little or no [^{32}P]LPA in resting platelets (Figure 4). Previous mass determinations indicate a net production of about 5 ng of LPA/10^9 platelets (i.e. approx. 12 pmol/10^9 platelets) upon stimulation with thrombin for 2 min [17]. Our analysis of LPA in cells and supernatants reveals that as much as 90% of newly generated LPA is released into the medium (Table 1). Based on the aforementioned mass determinations, this would amount to roughly 10 pmol of LPA released per 10^9 cells. In blood clots, however, the local LPA concentrations are expected to attain much higher levels.

LPA formation and release by thrombin-activated platelets is inhibited in the presence of EDTA (Figure 5) (cf. [16]). In the nominal absence of bivalent cations, platelets are unable to aggregate due to impaired fibrinogen binding to the platelet

Table 1 Formation and release of PA and LPA in activated platelets

Data shown are derived from the experiment in Figure 4. Two other experiments gave qualitatively similar results. For the 'd.p.m.' values, radioactivity in individual phospholipids was determined, and normalized to total [^{32}P]P_i-labelled phospholipids as described in the Materials and methods section: *not significantly different from background values; ND, not determined.

	Unstimulated			Thrombin-stimulated		
	Cells (d.p.m.)	Medium (d.p.m.)	Ratio medium/ cells	Cells (d.p.m.)	Medium (d.p.m.)	Ratio medium/ cells
LPA	120*	66*	ND	198	2648	13.4
PA	1403	230	0.16	4543	331	0.07
PC	2034	340	0.17	1678	161	0.09
PI	5413	772	0.14	5216	568	0.11

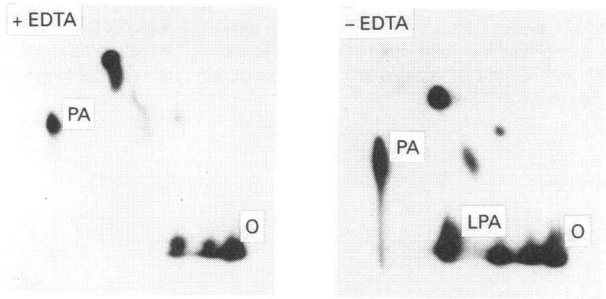

Figure 5 Inhibitory effect of EDTA on LPA release

Platelets were labelled with [^{32}P]P_i and stimulated for 10 min with thrombin, in either the absence or the presence of 10 mM EDTA as indicated. Medium was collected by centrifugation, extracted and analysed on two-dimensional t.l.c. as detailed in the Materials and methods section. LPA and PA spots were identified by co-migration with unlabelled markers; O, origin. Appearance of lipids other than LPA in the supernatant is attributable to residual membrane debris (cf. Figure 4).

surface [32]. Whether aggregation itself is a prerequisite for LPA production, or else the conversion of PA into LPA via phospholipase A_2 [18] depends on Ca^{2+} influx, remains to be explored.

Conclusions

The present study advances our understanding of LPA as a bioactive phospholipid in that it demonstrates that LPA is rapidly released from activated platelets and that LPA is an active albumin-bound constituent of serum. In view of LPA's mitogenic potential, we propose that platelet-derived LPA participates in the natural wound-healing process by stimulating cell proliferation at sites of injury and inflammation, probably in synergy with other platelet-derived mediators and peptide growth factors. In addition to activating quiescent fibroblasts, LPA acts on platelets themselves, which may introduce an element of signal amplification (positive feedback) in the initial aggregation response, perhaps similar to the mechanism by which secreted arachidonate metabolites and ADP amplify platelet responses [32]. It remains to be determined whether LPA is released via a distinct secretory pathway, or simply diffuses out of the cells during the irreversible phase of aggregation when platelets become leaky. Further experiments are also required to assess whether activated platelets are the only source of serum-borne LPAs. Given the great diversity of LPA-responsive cell types, ranging from amphibian oocytes [33–35] to mammalian neuronal cells [7], we expect that LPA formation and release would not be restricted to activated platelets. Support for this view is provided by a recent study which indicates that LPA is rapidly generated in growth-factor-stimulated fibroblasts (see Table 2 in [36]). The possibility that, under certain conditions, cell types other than platelets also secrete LPA into the extracellular space is now under investigation.

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